Osteopontin in cerebrospinal fluid as diagnostic biomarker for central nervous system lymphoma

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Abstract: Central nervous system lymphoma (CNSL) is diagnostically challenging. The identification of reliable and easy to measure biomarkers is desirable to facilitate diagnosis. Here, we evaluated the value of cerebrospinal fluid (CSF) osteopontin (OPN) as a diagnostic biomarker for CNSL. OPN concentrations in CSF from 37 patients with CNSL (29 with primary CNSL and 8 with secondary CNS involvement of systemic lymphoma) and 36 controls [6 patients with inflammatory CNS disease other than multiple sclerosis (MS), 8 with MS, 9 with glioblastoma (GBM) and 13 healthy controls] were determined using an enzyme-linked immunosorbent assay. Non-parametric tests and receiver operating characteristic (ROC) curves were performed for determination of diagnostic accuracy. Median CSF OPN level in all CNSL patients was 620 ng/mL and higher than in patients with inflammatory CNS disease (356 ng/mL); $P < .05$, MS (163 ng/mL); $P < .01$, GBM (41 ng/mL); $P < .01$, or healthy controls (319 ng/mL); $P < .01$. The area under the ROC curve was 0.865 [95 % confidence interval (CI) 0.745-0.985] for differentiating CNSL and patients with inflammatory CNS disease; 0.956 (95 % CI 0.898-1.000) for CNSL and MS patients; 0.988 (95 % CI 0.964-1.000) for CNSL and GBM patients, and 0.915 (95 % CI 0.834-0.996) for CNSL patients and healthy controls. In multivariate analysis, high CSF OPN level was associated with shorter progression-free (HR 1.61, 95 % CI 1.13-2.31; $P = .009$) and overall survival (HR 1.52, 95 % CI 1.04-2.21; $P = .029$). CSF OPN is a potential biomarker in CNSL.

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Osteopontin in cerebrospinal fluid as diagnostic biomarker for central nervous system lymphoma

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Abstract

Background: Central nervous system lymphoma (CNSL) is diagnostically challenging. The identification of reliable and easy to measure biomarkers is desirable to facilitate diagnosis. Here, we evaluated the value of cerebrospinal fluid (CSF) osteopontin (OPN) as a diagnostic biomarker for CNSL.

Methods: OPN concentrations in CSF from 37 patients with CNSL (29 with primary CNSL and 8 with secondary CNS involvement of systemic lymphoma) and 36 controls (6 patients with inflammatory CNS disease other than multiple sclerosis [MS], 8 with MS, 9 with glioblastoma [GBM] and 13 healthy controls) were determined using an enzyme-linked immunosorbent assay. Non-parametric tests and receiver operating characteristic (ROC) curves were performed for determination of diagnostic accuracy.

Results: Median CSF OPN level in all CNSL patients was 620 ng/mL and higher than in patients with inflammatory CNS disease (356 ng/mL); $P < .05$, MS (163 ng/mL); $P < .01$, GBM (41 ng/mL); $P < .01$, or healthy controls (319 ng/mL); $P < .01$. The area under the ROC curve was .865 (95% confidence interval [CI] .745-.985) for differentiating CNSL and patients with inflammatory CNS disease; .956 (95% CI .898-1.000) for CNSL and MS patients; .988 (95% CI .964-1.000) for CNSL and GBM patients, and .915 (95% CI .834-.996) for CNSL patients and healthy controls. In multivariate analysis, high CSF OPN level was associated with shorter progression-free (HR 1.61, 95% CI 1.13-2.31, $P = .009$) and overall survival (HR 1.52, 95% CI 1.04-2.21; $P = .029$).

Conclusions: CSF OPN is a potential biomarker in CNSL.

Introduction

Central nervous system lymphoma (CNSL) arises as primary CNS lymphoma (PCNSL) – a lymphoma that is confined to the CNS at time of diagnosis – or as secondary CNS involvement of systemic lymphoma (SCNSL) [1-3]. The diagnosis is typically based on contrast-enhanced cranial magnetic resonance imaging and histopathological confirmation by a biopsy from a CNS lesion [4]. However, a biopsy may be challenging in vulnerable brain regions, and in some cases histological findings are inconclusive, particularly after steroid pre-treatment. The utility of cerebrospinal fluid (CSF) diagnostics based on tumour cell identification such as conventional CSF cytomorphology, flow cytometry [5] or monoclonality assessment by polymerase chain reaction [6] is limited to cases with leptomeningeal involvement which is present in approximately 40-50% of SCNSL and in less than 20% of PCNSL [7, 8]. Identification of reliable diagnostic markers in CSF representing an easily accessible compartment is highly desirable to facilitate the diagnosis.

In the search for diagnostic and prognostic biomarkers for CNSL, microRNA expression patterns in the CSF and blood have yielded promising results [9-11]. These studies, however, lacked a comparison to other brain tumours. Sasayama *et al.* reported elevated interleukin-10 in CSF of PCNSL patients as compared to patients with other CNS malignancies [12]. Our group detected elevated CXCL13 concentration in CSF of 30 PCNSL patients as compared to patients with other CNS malignancies and non-malignant CNS diseases [13]. These findings were confirmed in an analysis of 60 PCNSL and 23 SCNSL samples [14]. Viaccoz *et al.* identified neopterin, a nonspecific marker of CNS inflammation, as possible marker to differentiate PCNSL from other brain tumours [15].

Osteopontin (OPN) is a proinflammatory cytokine and cell-matrix glycoprotein with numerous cellular functions including immune cell activation, B-cell migration and proliferation, chemotaxis, cell communication, focal adhesion, and suppression of antitumour immunity [16]. High OPN expression is associated with progression, metastatic spread, and poor prognosis in many malignancies. Several studies in different human malignancies identified OPN as suitable diagnostic and prognostic biomarker [17-24]. In PCNSL, *osteopontin* (also known as *secreted phosphoprotein 1, SPP1*) is one of the most differentially up-regulated genes as compared to both nodal and extranodal non-CNS diffuse large B-cell lymphoma (DLBCL) [25-27]. Immunohistochemical analyses of

formalin-fixed, paraffin-embedded tissue previously confirmed high-level expression of OPN in PCNSL [25, 26, 28]. In this study, we evaluated the utility of OPN levels in the CSF as a diagnostic biomarker for CNSL.

Materials and Methods

Patients and samples

CSF samples were obtained prior to chemotherapy by lumbar puncture from 37 immunocompetent patients with CNSL: 29 with PCNSL and 8 with SCNSL. Steroid pre-treatment was allowed. Four control groups were analysed: (1) 6 patients with inflammatory CNS disease other than multiple sclerosis (MS), among them cerebral toxoplasmosis, cerebral sarcoidosis, and unspecified non-infectious encephalitis, (2) 8 patients with MS, (3) 9 patients with glioblastoma (GBM) and (4) 13 healthy controls, i.e. patients without CNS disease who underwent diagnostic lumbar puncture for peripheral neuropathy or headache, the latter ones to rule out meningitis or subarachnoidal bleeding; all of those showed no evidence of CNS disease in the following workup.

All CSF samples were processed immediately at room temperature by centrifugation for four minutes at 4,000 rpm. Supernatants were collected and stored at -80°C. Paired serum samples were collected from 17 PCNSL patients, all inflammatory CNS disease patients, and all healthy controls at the time of lumbar puncture and stored at -80°C. CSF cell count and protein were analysed in 26 CNSL patients, all inflammatory CNS disease patients, and all healthy controls.

All patients gave written informed consent in accordance with the Declaration of Helsinki and the local Institutional Review Board-approved protocols.

Enzyme-linked immunosorbent assay

OPN concentrations in CSF and serum were determined using the enzyme-linked immunosorbent assay (ELISA) from R&D (Quantikine ELISA Human Osteopontin Immunoassay, DOST00; R&D Systems Europe, Ltd., Abingdon, UK).

Statistical analyses

Descriptive and explorative data analysis of clinical and ELISA data were performed. Quantitative parameters were tested for normal distribution. Distribution of patients' characteristics was analysed by the chi-square test. The Kruskal-Wallis and Mann-Whitney U tests with adjusted *P* value due to Bonferroni correction were used as non-parametric tests to evaluate the quantitative parameters. If not stated different, Bonferroni correction was applied with factor 10 (number of pairwise comparisons between 5 groups) in analyses with CNSL patients (PCNSL and SCNSL) vs. control groups. Bonferroni correction with factor 15 (number of pairwise comparisons between 6 groups) was applied in analyses with PCNSL vs. SCNSL vs. control groups. In OPN serum, CSF cell count, and CSF protein analyses Bonferroni correction with factor 3 (number of pairwise comparisons between 3 groups) was used. Comparisons between CSF and serum levels in identical patients were done using the Wilcoxon signed rank test. Receiver operating characteristic (ROC) curves were performed on levels of CSF OPN. In these curves, for each potential cut-off value of the respective diagnostic marker the pair of sensitivity and 1-specificity is determined and plotted vs. each other. The cut-off values themselves cannot be read from the curve. The choice of the specific cut-off was done according to the criterion of the OPN level at which sensitivity and specificity were most alike. Overall survival (OS) and progression-free survival (PFS) were analysed with the Kaplan-Meier-method for PCNSL patients. PFS was defined as the time from beginning of treatment to first progression or death from any cause. OS was defined as the time from beginning of treatment to death. Spearman correlations (Spearman correlation coefficient rho = r) were used to quantify the association of multifocal brain involvement (3 ordered categories) and CSF OPN level, age and CSF OPN level, CSF cell count and CSF OPN level as well as CSF protein and CSF OPN level. To assess the prognostic value of CSF OPN in principle, simple and multiple Cox proportional hazard models were calculated ("forward" stepwise variable selection with criteria $P = .10$ (exclusion), $P = .05$ (inclusion)). All hazard ratios (HR) refer to a change of 100 units of CSF OPN.

For statistical analyses IBM SPSS software package, release 22.0, and GraphPad Prism software package, release 6.0, were used.

Results

Patient characteristics

Clinical and histopathological characteristics of CNSL patients are summarised in Table 1. Twenty-three (79%) PCNSL and five (63%) SCNSL patients had steroid treatment before lumbar puncture.

An elevated (≥5/μL) CSF cell count was detected in 16 of 26 (62%) patients with CNSL (median 10.0/μL, range 1.0-14,842.0/μL); total CSF protein was elevated (>45 mg/dL) in 20 (77%) patients (median 74.0 mg/dL, range 28.0-341.0 mg/dL). CSF cell count was elevated in one of six (17%) patients with inflammatory CNS disease (median 1.7/μL, range 0.3-9.3/μL) and in none of 13 healthy controls (median 0.7/μL, range 0.3-2.7/μL). Total CSF protein was elevated in 3 (50%) cases with inflammatory CNS disease (median 48.1 mg/dL, range 40.2-64.5 mg/dL) and in one (8%) of the healthy controls (median 37.5 mg/dL, range 18.4-49.5 mg/dL).

CSF cell count was higher in CNSL patients than in patients with inflammatory CNS disease; $P < .01$ and healthy controls; $P < .01$. CSF protein in CNSL patients and patients with inflammatory CNS disease showed not significant difference; $P = .291$, but was higher in CNSL patients than in healthy controls; $P < .01$.

OPN levels in CNSL patients and control groups

CSF and serum OPN levels of CNSL patients and control groups as well as corresponding *P* values are shown in Figure 1, 2A, and 2B.

CSF OPN levels did not differ between PCNSL (median 620 ng/mL, range 60-890 ng/mL) and SCNSL patients (median 608 ng/mL, range 153-818 ng/mL); $P = 1.00$. According to the Kruskal-Wallis test there was a significant difference between the OPN CSF values in CNSL patients and all control groups; $P < .001$. Highest CSF OPN levels were found in PCNSL and SCNSL patients.

Patients with inflammatory CNS disease (median 356 ng/mL, range 253-531 ng/mL) and healthy controls (median 319 ng/mL, range 142-430 ng/mL) showed intermediate CSF OPN levels. Lowest CSF OPN levels were seen in MS (median 163 ng/mL, range 29-370 ng/mL) and GBM (median 41 ng/mL, range 22-191 ng/mL) patients. The median CSF OPN level in all CNSL patients was 620

ng/mL (range 60-890 ng/mL) and thus higher than in patients with inflammatory CNS disease; $P < .05$, with MS; $P < .01$, with GBM; $P < .01$ and healthy controls; $P < .01$ (Figure 1).

In contrast to CSF, median OPN serum levels were low and did not differ between PCNSL patients (median 52 ng/mL, range 17-231 ng/mL), patients with inflammatory CNS disease (median 99 ng/mL, range 40-273 ng/mL), and healthy controls (median 59 ng/mL, range 22-203 ng/mL); $P = .158$ (Figure 2A). The CSF/serum-ratio was predictably higher in PCNSL patients than in patients with inflammatory CNS disease or in healthy controls; both $P < .01$ (Figure 2B).

Steroid pre-treatment in CNSL patients did not influence CSF OPN level ($P = .984$). Spearman correlation analysis indicates a significant increase of CSF OPN with age in the healthy controls ($r = .663$, $P = .014$, $n = 13$), whereas in CNSL patients we did not find such a correlation ($r = -.186$, $P = .269$, $n = 37$). CSF OPN did not correlate with CSF cell count ($r = -.050$ in healthy controls and $r = -.179$ in CNSL). Moreover, CSF OPN weakly correlated with total CSF protein in CNSL patients ($r = .383$, $P = .053$), but not in healthy controls ($r = .209$, $P = .494$).

Diagnostic value of CSF OPN

On the basis of ROC curves, we analysed the diagnostic value of CSF OPN as a diagnostic biomarker for CNSL (Figure 3 and Supplementary Figure 1). The area under the ROC curve was .934 (95% confidence interval [CI] .873 - .995) for differentiating CNSL from all control patients (cut-off 400 ng/mL, sensitivity 87%, specificity 86%), .865 (95% CI .745 - .985) for differentiating CNSL from inflammatory CNS disease (cut-off 438 ng/mL, sensitivity 84%, specificity 83%), .956 (95% CI .898 - 1.000) for differentiating CNSL from MS (cut-off 362 ng/mL, sensitivity 89%, specificity 88%), .988 (95% CI .964 - 1.000) for CNSL vs. GBM patients (cut-off 240 ng/mL, sensitivity 95%, specificity 100%), and .915 (95% CI .834 - .996) for CNSL vs. healthy controls (cut-off 419 ng/mL, sensitivity 87%, specificity 85%).

Prognostic value of CSF OPN in patients with PCNSL

Survival data of 25 PCNSL patients with CSF OPN level measured at time of diagnosis were analysed. The median follow-up was 21.4 months. Median PFS and median OS were 26.5 months

(95% CI 14.4-38.5 months) and 32.5 months (95% CI 19.2-45.7 months), respectively. In univariate analysis, higher level of CSF OPN was significantly associated with shorter PFS (HR 1.47, 95% CI 1.02-2.10, *P* = .037), whereas the difference in OS was not significant (HR 1.28, 95% CI .89-1.84; *P* = .187, see also Supplementary Figure 2 with median CSF OPN as cut-off). In multivariate analysis with clinical characteristics age and sex, CSF OPN was significantly associated with shorter PFS (HR 1.61, 95% CI 1.13-2.31, *P* = .009) and OS (HR 1.52, 95% CI 1.04-2.21; *P* = .029). Spearman correlation analysis revealed a significant correlation between CSF OPN level and multifocal brain involvement (r = .460, *P* = .021).

Discussion

We provide first evidence that CSF OPN might be a valuable, easy to measure, and cost-effective diagnostic and prognostic biomarker in CNSL. ROC analyses revealed that CSF OPN discriminated patients with CNSL from control groups with high sensitivity and specificity. In contrast to elevated OPN in CSF, serum levels were low and comparable to those of patients with inflammatory CNS disease and healthy controls. This indicates that in CNSL patients, high CSF OPN levels are due to production and secretion in the CNS and are not the consequence of a systemic inflammation with concomitant CNS involvement.

Due to the rarity of CNSL, very little is known about the mechanisms leading to development of this disease in CNS, which is largely devoid of lymphoid tissue. High-levels of proinflammatory proteins such as neopterin [15] and - as described in this report - osteopontin may suggest a neuroinflammatory process. First data on gene and protein expression in PCNSL indicate an up-regulation of genes associated with cell signalling in extracellular matrix and adhesion related pathways such as DDR1, CXCL13, MUM1, TCL1a, CHI3L1 and OPN [25-28]. When OPN expression levels were compared between PCNSL, extranodal DLBCL, and nodal DLBCL, an expression was observed in 100% and 95% in the both first groups compared to 25% in the third group, suggesting its probable role in CNS tissue tropism [25]. Moreover, higher CSF OPN levels were found in childhood acute leukaemia with CNS involvement than in patients without CNS involvement suggesting a role of OPN as a marker of secondary CNS involvement [19]. Since high CSF OPN levels were detected in SCNSL patients, OPN

could represent an early marker of secondary CNS involvement in systemic lymphoma. This is worth further evaluation considering the low diagnostic value of currently known risk factors to identify patients with high risk for secondary CNS involvement in lymphoma [29] and an urgent need for markers guiding CNS prophylaxis.

Numerous studies reported increased CSF OPN levels in patients with MS and other neuroinflammatory and neurodegenerative diseases [30-33]. In our study, the median CSF OPN level of MS patients was in accordance with data published by others [31, 33]. However, CSF OPN level in patients with neuroinflammatory disease, e.g. MS, reflects the extent of inflammation and dynamically varies in correlation to activity of disease [30]. Therefore, the median CSF OPN level of our MS patients may differ to OPN levels reported by others.

For GBM, high-level OPN expression has been demonstrated by immunohistochemistry, and relatively high OPN levels in CSF of GBM patients were reported in one study [34], which is in contrast to our findings. Methodological differences as well as differences in patients' characteristics and pre-treatment might be explanations for the difference to our GBM samples. CSF OPN levels of our healthy controls were higher than reported by other studies of patients with childhood acute leukaemia without CNS involvement and patients with neurologic symptoms, but without confirmed neurologic disease [19, 35, 36]. Although no CNS disease was found in our healthy controls, we cannot rule out conditions, as e.g. peripheral neuropathy, with an influence on CSF OPN levels in some of them.

In addition to the diagnostic role, OPN may represent an easy to measure prognostic marker in CNSL. Patients with higher OPN levels had shorter PFS and OS. Furthermore, higher CSF OPN significantly correlated with multifocal brain involvement, which indicates that OPN level reflects tumour burden in CNSL. An association of multifocal brain involvement with inferior outcome has previously been reported [37, 38]. Moreover, a correlation of increased circulating OPN levels and increased OPN expression by tumour cells with an unfavourable prognosis was found in other human malignancies [20-22].

OPN and its receptor CD44 have been identified as a promising therapeutic target for cancer therapy [39, 40]. Considering the generally poor prognosis of CNSL, identifying new targets for more effective therapies is highly desirable in this disease. If the contribution of OPN to CNS lymphomagenesis is confirmed, evaluation of the inhibition of OPN and/or its receptor as novel treatment strategy for CNSL will be worth evaluating.

A comprehensive overview of previously reported potential biomarkers for PCNSL diagnosis was published recently [15]. A combination of OPN with other biomarkers such as IL-10, CXCL13 or neopterin could improve diagnostic yield and should be tested. The small sample size, especially of the control groups, represents a major limitation of our study. Further studies with larger control groups are needed to validate our results.

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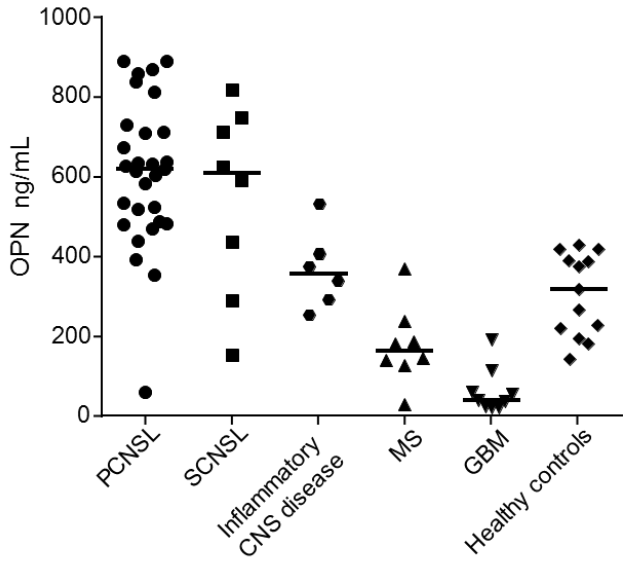
Figure 1. CSF OPN levels in CNSL and control patients.



Comparison	*P* value
PCNSL vs. SCNSL	= 1.00
CNSL vs. ICD	< .05
CNSL vs. MS	< .01
CNSL vs. GBM	< .01
CNSL vs. healthy controls	< .01
ICD vs. MS	= .05
ICD vs. GBM	< .01
ICD vs. healthy controls	= 1.00
MS vs. GBM	= .15
MS vs. healthy controls	= .08
GBM vs. healthy controls	< .01

Figure legend: Black lines indicate median OPN levels. *P* values were adjusted by Bonferroni correction for multiple comparisons. CSF...cerebrospinal fluid, OPN...osteopontin, CNSL...central nervous system lymphoma (primary and secondary), PCNSL...primary CNSL, SCNSL...secondary CNS involvement of systemic lymphoma, ICD…inflammatory CNS disease, MS…multiple sclerosis, GBM...glioblastoma. One of the PCNSL patient had considerably low CSF OPN level compared to all other PCNSL patients. In this patient, PCNSL had formerly been diagnosed and treated and CSF was obtained at time of relapse, which was assumed only by clinical and radiological characteristics. As histological results from time of relapse were not conclusive, we cannot definitely be certain of PCNSL diagnosis in this patient.

Figure 2. A) CSF and serum OPN levels in PCNSL and control patients, **B)** CSF/serum-ratio in PCNSL and control patients.

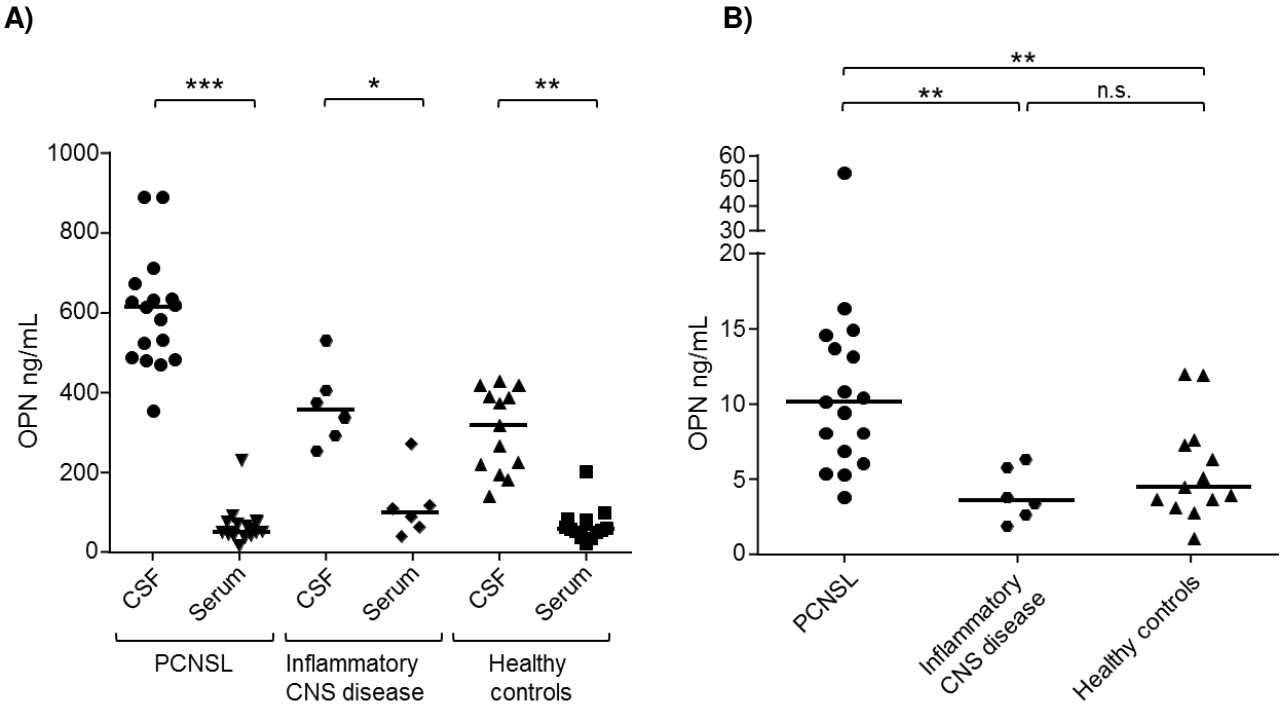


Figure legend: *…*P* < .05, **…*P* < .01, ***…*P* < .001, n.s….not significant. Black lines indicate median OPN levels. *P* values in Figure 2 B) were adjusted by Bonferroni correction for multiple comparisons. CSF...cerebrospinal fluid, OPN...osteopontin, PCNSL...primary central nervous system lymphoma, CNS…central nervous system.

Figure 3. ROC curve analyses of CSF OPN levels.

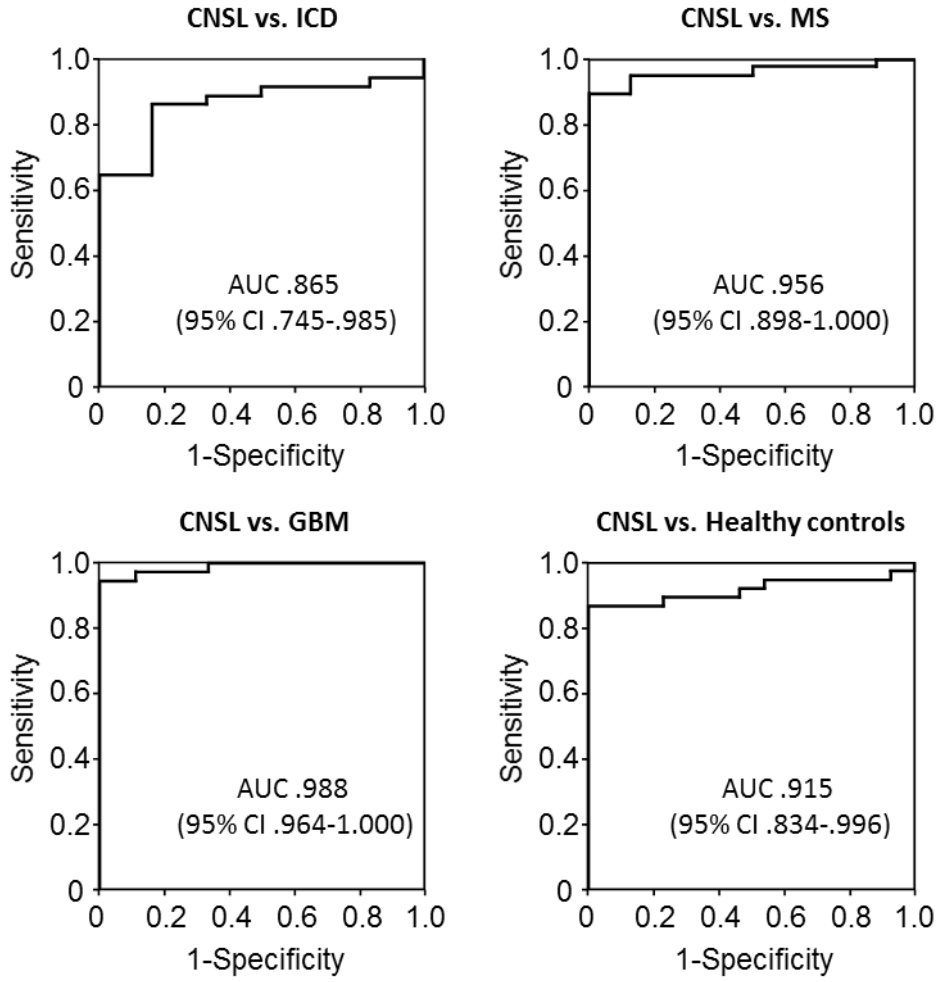


Figure legend: ROC…receiver operating characteristic, CSF...cerebrospinal fluid, OPN...osteopontin, CNSL...central nervous system lymphoma (primary and secondary), ICD…inflammatory CNS disease, MS…multiple sclerosis, GBM...glioblastoma, AUC…area under the curve, CI…confidence interval.

Table 1. Clinical characteristics in CNSL patients and control groups.

	CNSL (n=37)	PCNSL (n=29)	SCNSL (n=8)	ICD (n=6)	MS (n=8)	GBM (n=9)	Healthy controls (n=13)
Median age, years (range)	65 (43-84)	65 (43-84)	66 (47-75)	58 (42-69)	45 (31-60)	58 (45-75)	53 (23-81)
Males (%)	17 (46)	13 (45)	4 (50)	3 (50)	3 (38)	5 (56)	8 (62)
Histopathological diagnosis (%)							
DLBCL	32 (86)	27 (93)	5 (63)	n.a.	n.a.	n.a.	n.a.
Mantle cell lymphoma	1 (3)	0	1 (13)	n.a.	n.a.	n.a.	n.a.
Follicular lymphoma	1 (3)	0	1 (13)	n.a.	n.a.	n.a.	n.a.
Other low-grade B-cell lymphoma	1 (3)	0	1 (13)	n.a.	n.a.	n.a.	n.a.
B-cell lymphoma without further specification	1 (3)	1 (4)	0	n.a.	n.a.	n.a.	n.a.
No conclusive histological results	1 (3)	1 (4)	0	n.a.	n.a.	n.a.	n.a.
Localisation of CNS manifestation (%)							
Brain parenchyma only	32 (86)	27 (93)	5 (63)	n.a.	n.a.	n.a.	n.a.
Brain parenchyma and eye	2 (5)	2 (7)	0	n.a.	n.a.	n.a.	n.a.
Brain parenchyma and CSF	1 (3)	0	1 (13)	n.a.	n.a.	n.a.	n.a.
CSF only	2 (5)	0	2 (25)	n.a.	n.a.	n.a.	n.a.
CSF cell count	n=26	n=22	n=4	n=6	n.d.	n.d.	n=13
Elevated CSF cell count (≥5/µL) (%)	16 (62)	13 (59)	3 (75)	1 (17)	n.d.	n.d.	0
Median CSF cell count, /µL (range)	10 (1-14.842)	9 (1-230)	44 (4-14.842)	2 (0-9)	n.d.	n.d.	1 (0-3)
CSF protein	n=26	n=22	n=4	n=6	n.d.	n.d.	n=13
Elevated CSF protein (>45 mg/dL) (%)	20 (77)	16 (73)	4 (100)	3 (50)	n.d.	n.d.	1 (8)
Median CSF protein, mg/dL (range)	74 (28-341)	62 (28-341)	204 (76-258)	48 (40-65)	n.d.	n.d.	38 (18-50)

Table legend: CNSL…central nervous system lymphoma, DLBCL... diffuse large B-cell lymphoma, CSF...cerebrospinal fluid, PCNSL...primary central nervous system lymphoma, SCNSL...secondary CNS involvement of systemic lymphoma, ICD…inflammatory CNS disease, MS…multiple sclerosis, GBM...glioblastoma n.a.…not applicable, n.d….not done.

Supplementary Figure 1. ROC curve analysis of CSF OPN levels (CNSL vs. all control groups).

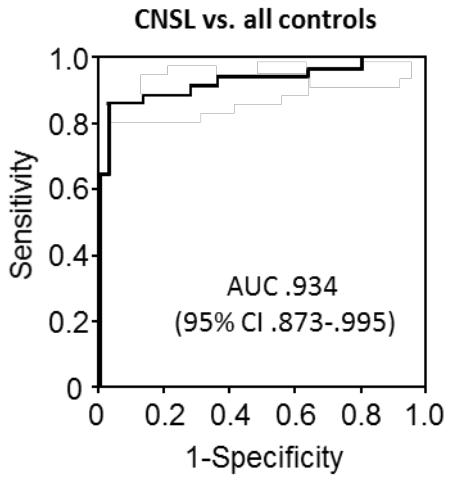


Figure legend: ROC…receiver operating characteristic, CSF...cerebrospinal fluid, OPN...osteopontin, CNSL...central nervous system lymphoma (primary and secondary), AUC…area under the curve, CI…confidence interval.

Supplementary Figure 2. Comparison of progression-free survival and overall survival according to CSF OPN expression (cut-off = median CSF OPN).